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A stromal gene signature associated with inflammatory breast cancer

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The factors that determine whether a breast carcinoma will develop into inflammatory breast cancer (IBC) remain poorly understood. Recent evidence indicates that the tumor stroma influences cancer phenotypes. We tested the hypotheses that the gene expression signature of the tumor stroma is a distinctive feature of IBC. We used laser capture microdissection to obtain enriched populations of tumor epithelial cells and adjacent stromal cells from 15 patients with IBC and 35 patients with invasive, noninflammatory breast cancer (non-IBC). Their mRNA expression profiles were assessed using Affymetrix GeneChips™. In addition, a previously established classifier for IBC was evaluated for the resulting data sets. The gene expression profile of the tumor stroma distinguished IBC from non-IBC, and a previously established IBC prediction signature performed better in classifying IBC using the gene expression profile of the tumor stroma than it did using the profile of the tumor epithelium. In a pathway analysis, the genes differentially expressed between IBC and non-IBC tumors clustered in distinct pathways. We identified multiple pathways related to the endoplasmic stress response that could be functionally significant in IBC. Our findings suggest that the gene expression in the tumor stroma may play a role in determining the IBC phenotype.

Key words: inflammatory breast cancer; stroma; gene signature; prediction

Introduction

Inflammatory breast cancer (IBC) is a rapidly progressing breast cancer subtype associated with clinical symptoms such as skin discoloration, erythema and peau d'orange, and is accompanied by a high mortality rate.[1,2] Although the symptoms resemble the presence of acute inflammation, the term "IBC" is a misnomer. Inflammation has not been found to be causal in IBC, and host inflammatory cells are generally not more common in the tumor stroma of IBC patients than in other breast cancer subtypes.[2] The poor survival associated with IBC is multifactorial, relating to rapid disease progression, late diagnosis, poorly defined tumor margins and a high tumor-vessel density, which combined lead to locally advanced disease and distant metastasis.[2–4] The factors that promote the development of IBC are still elusive despite efforts to identify the key genes involved in IBC.[5–8] Unlike most non-IBC tumors, the tumors of IBC patients maintain the expression of E-cadherin,[8,9] thus preserving cell–cell binding integrity. As a result, the main mechanism of IBC invasion involves tumor emboli as opposed to single cell invasion.

The analysis of microdissected tumor stroma from colon cancer patients has demonstrated that the expression of genes contributing to invasion and metastasis is altered in stromal cells.[10] Gene expression in the stroma adjacent to tumor cells is influenced by both host genetics and tumor–stroma interactions. Such interactions appear to be crucial for cancer progression[11] and can induce an invasive phenotype in human breast cancer cells.[12] Host genetics may play an equally important role. For example, fibroblasts from relatives of patients with familial breast cancer more frequently show an abnormal migratory behavior and a tumor-like phenotype than do fibroblasts from donors without such a family history.[13] Others have found evidence that allelic diversity in the host genetic background is a determinant of tumor metastasis in mice.[14] Thus, the intrinsic gene expression profile of the tumor stroma may strongly influence a cancer's phenotype, aggressiveness and outcome.

In the present study, the hypothesis was pursued that the gene expression signature of the tumor stroma is a distinctive feature of IBC. We also investigated whether a previously established classifier for IBC can distinguish between IBC and non-IBC tumors with gene signatures obtained from microdissected samples, *e.g.*, tumor epithelium and tumor stroma. We used laser capture microdissection (LCM) to obtain samples enriched in tumor epithelium and tumor stroma from 15 IBC and 35 invasive, noninflammatory breast cancer (non-IBC) cases to study the relative contribution of each component to the IBC phenotype. All previous studies of IBC have used bulk tumor samples. Downsides of this approach include dilution of gene expression signatures from any one tissue subcompartment and the inability to distinguish the separate roles of the different subcompartments. In particular, the significance of the stromal gene signature in IBC is obscured by this approach.

Material and methods

Collection of tissue specimens and survival data

We examined 52 fresh-frozen, excised breast tissues obtained from the Department of Pathology at the University of Maryland. Of the tissues, 50 were from primary tumors of breast cancer patients who received surgery, and 2 were from noncancerous tissues collected during reduction mammoplasties. The specimens were collected between 1993 and 2003 at hospitals in the greater Baltimore area, Maryland, as part of a larger study to examine molecular markers associated with breast cancer development and progression. The recruitment procedures, patients' eligibility and the determination of breast cancer survival for this population have been described in detail.[15] Information on therapy was abstracted from the medical records. Twenty-three patients in the study received neoadjuvant therapy; all of those received chemotherapy with 3 patients (2 IBC, 1 non-IBC) receiving a combination of chemotherapy and radiation. The Institutional Review Boards at the participating institutions approved the study.

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Clinicopathology

Information on tumor histology and disease staging was abstracted from the pathology reports. Disease staging was performed according to the tumor-node-metastasis (TNM) system according to the American Joint Committee on Cancer (AJCC)/Union Internationale Contre le Cancer (UICC) classification. Fifteen of the 50 tumors were selected, because they had previously been identified as possible IBC cases based on tumor pathology. The diagnosis of IBC was confirmed by reviewing the pathology and medical reports. Six patients had IBC based on pathology as indicated by dermal lymphatic invasion with tumor emboli and the involvement of skin; 9 patients carried the pathological diagnosis with additional clinical signs of IBC such as erythema and peau d'orange. Thus, we used a conservative diagnostic definition of IBC that has dermal lymphatic invasion as the common criterion. This classification has been used in other studies of IBC.[6,16] To further evaluate our IBC classification, we compared the gene expression profiles of tumors that had the pathological diagnosis of IBC with those of tumors that had both the pathological and clinical diagnosis of IBC. We could not find any genes in the microdissected tumor epithelium and tumor stroma that were significantly differently expressed between these 2 groups at any FDR cutoff. We concluded that these 2 patient groups are very similar in terms of their tumor gene expression profiles.

Laser capture microdissection

Frozen 8-μm serial sections from OCT-preserved frozen tissues were prepared and mounted on plain, uncharged microscope slides. One Hematoxylin/eosin-stained section of each specimen was reviewed by a pathologist to confirm diagnosis and presence of tumor before commencing dissection. LCM was performed at the NIH Collaborative Research LCM Core Laboratory with the Pixcell II LCM system (Arcturus, Mountain View, CA). We collected tumor and stromal samples from tumor specimens and epithelial cells and stroma from the breast reduction tissues. At least 3,000–5,000 cells were obtained per specimen.

RNA isolation and labeling

Total RNA was isolated using the PicoPure protocol (Arcturus, Mountain View, CA). RNA quality was verified using the RNA 6000 Pico LabChip on a 2100 Bioanalyzer (Agilient, Palo Alto, CA). The mRNA was amplified and labeled using the Small Sample Labeling Protocol vII from Affymetrix (Santa Clara, CA). Briefly, biotin-labeled cRNA was generated with 2 linear amplification steps by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX), followed by the labeling step using the BioArray HighYield RNA Transcript Labeling Kit T3 from Enzo Life Sciences (Farmingdale, NY). This method has previously been evaluated[17] and does not introduce a bias into the computed relative gene expression values. Labeled cRNA was hybridized onto the Affymetrix HG-U133A GeneChips (#900366) according to manufacturer's protocol. The HG-U133A array contains 22,283 probe sets that match transcripts of approximately 13,000 human genes. In accordance with Minimum Information About a Microarray Gene Experiment (MIAME) guidelines, CEL files of the microarray data, the normalized expression data and additional patient information were deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/). The submission of this information was reviewed and approved by the IRB. The GEO submission accession number is GSE5847.

Analysis of gene expression data

All chips were normalized using the robust multichip analysis procedure (www.bioconductor.org). Gene lists comparing mRNA expression in IBC stroma with that in non-IBC stroma and IBC tumor with that in non-IBC tumor were generated using moderated *t*-scores. *p* values for the moderated *t*-statistic were determined by the standard method of permuting samples to obtain a null distribution. The moderated *t* statistic is very similar to the statistic that is used by SAM[18] or by the BioConductor's eBayes procedure. We report FDR-controlled *p* values.

Prediction analysis

To evaluate our classification of samples, we used prediction analysis for microarrays (PAM) to classify patients as either IBC or non-IBC using a previously validated signature for IBC.[6] PAM was applied to classify our dataset according to the predefined IBC signature for the intended categories.[19] In this analysis, the threshold delta was chosen based on the best compensation for both training error rates and coefficient of variation (CV) error rates. Cross validation was performed leaving out 10% of the samples to determine the appropriate threshold parameter in PAM.

Pathway analysis

The analysis of pathway and network data was performed using in-house software[20] and the Ingenuity (Ingenuity Systems, Redwood City, CA) and MetaCore™ (GeneGO, St. Joseph, MI) pathway analysis tools. Biological networks identified by the programs were assessed using Gene Ontology Biological Processes (GOBP) classification (http://www.geneontology.org) and BioCarta mappings (http://www.biocarta.com/genes/allPathways.asp). We analyzed and graphically displayed in heatmaps all GOBP terms and BioCarta pathways that have an enrichment of differentially expressed genes in tumor stroma and epithelium for 3 contrasts, (*i*) IBC *versus* non-IBC, (*ii*) estrogen receptor α (ER)-positive *versus* ER-negative tumors and (*iii*) cyclin E-positive *versus* cyclin E-negative tumors. For significance analysis of biological themes at the GOBP term or BioCarta pathway level, resulting gene lists were subjected to a one-sided Fisher's exact test. The test assessed whether the enrichment of differently expressed genes in a GOBP term or BioCarta pathway was statistically significant for a given comparison, *e.g.*, IBC stroma *versus* non-IBC stroma, at the $p < 0.05$ level.

Immunohistochemistry

Laminin-5, γ-2 chain (*LAMC2*), protein expression was evaluated in IBC tumors. Formalin-fixed and paraffin-embedded 5 μm slides were processed as described.[21] After antigen retrieval and blockage of the endogenous peroxidase, protein expression was evaluated using the following primary antibody: 1:100 diluted mouse monoclonal anti-human laminin-5, γ-2 chain, antibody (M7262; clone 4G1; DakoCytomation).

Results

Microdissection

LCM successfully yielded the gene expression profiles for both tumor epithelium and adjacent stroma of 48 cases, respectively. In 2 cases of IBC, LCM did not provide sufficient amounts of good quality total RNA from the tumor epithelium. In another case of IBC and 1 case of non-IBC, sufficient total RNA could not be isolated from the tumor stroma. The clinical characteristics of the patients are shown in Table I. Fifteen were classified as patients with IBC based on pathology and clinical presentation of the disease. The other 35 had invasive breast cancer classified as non-IBC.

We did not stage-match the IBC and non-IBC patients. Instead, we over-sampled for non-IBC patients with ER-negative tumors and poor disease outcome (less than 5 year survival) to achieve matching and minimize confounding. Using those matching criteria, we found that ER mRNA expression was not significantly different when comparing IBC and non-IBC tumor epithelium. For comparison, the ER mRNA expression was highly significantly different in the microdissected tumor epithelium comparing immunohistochemically determined ER-positive tumors with the ER-negative tumors in our study (ratio: 8.04 for *ESR1* probeset 205225_at with ER-negative as the reference; *p* value: < 0.001 by moderated *t*-test).

TABLE 1 – CLINICAL CHARACTERISTICS OF BREAST CANCER PATIENTS

Characteristic	All cases ($n = 50$)	IBC ($n = 15$)	Non-IBC ($n = 35$)	p value[1] IBC versus non-IBC
Age at diagnosis				
Mean ± SD[2] (years)	57 ± 15	53 ± 13	58 ± 16	0.26
ER status				
Positive	22 (44%)	6 (40%)	16 (46%)	
Negative	27 (54%)	9 (60%)	18 (51%)	0.76
Unknown	1 (2%)	0 (0%)	1 (3%)	
Cyclin E status				
Positive	22 (44%)	7 (47%)	15 (43%)	
Negative	22 (44%)	5 (33%)	17 (49%)	0.74
Unknown	6 (12%)	3 (20%)	3 (9%)	
TNM Stage[3]				
I	4 (8%)	0 (0%)	4 (11%)	
II	30 (60%)	1 (7%)	29 (83%)	<0.0001
≥III	16 (32%)	14 (93%)	2 (6%)	
Histology				
Lobular	5 (10%)	1 (7%)	4 (11%)	
Ductal[4]	44 (88%)	14 (83%)	30 (77%)	0.73
Other	1 (2%)	0 (0%)	1 (3%)	
Median survival (months)	44	21	55	
Range	3–132	3–104	12–132	0.007
Neoadjuvant therapy				
No	28 (56%)	1 (7%)	27 (77%)	
Yes	21 (42%)	13 (87%)	8 (23%)	0.0001
Unknown	1 (2%)	1 (7%)	0 (0%)	

Two-sided *t*-test for age; two-sided Fisher's exact test for ER and cyclin E status (positive *vs*. negative), and neoadjuvant therapy (no *vs*. yes), TNM stage and histology; two-sided Wilcoxon rank sum test for survival by months. ER status and cyclin E expression in the tumor samples were scored as described previously.[15]

[1]IBC versus non-IBC.–[2]SD, standard deviation.–[3]Pathology report.–[4]Ductal and mixed ductal/lobular.

Gene expression analysis

As most IBC patients had received neoadjuvant chemotherapy, we also investigated the possible confounding effect of such therapy on gene expression. The effect of neoadjuvant therapy could not be studied within the IBC cohort. However, it was possible to compare the gene expression profiles of both tumor epithelium and tumor stroma from IBC patients who received neoadjuvant therapy with those of the tumor epithelium and tumor stroma from non-IBC patients who also received neoadjuvant therapy. This analysis generated only modest differences relative to the contrasts between IBC tumors and non-IBC tumors without the stratification by neoadjuvant therapy (data not shown). We also compared non-IBC tumors from patients who had or had not received neoadjuvant therapy. Few gene differences were found between the 2 therapy groups, which is consistent with previous observations in other breast cancer studies. Of the 17 genes that were previously identified as being altered in breast tumors post chemotherapy,[22] only 1 was significantly differently expressed between IBC tumors and non-IBC tumors in our study. Because of these results, we concluded that chemotherapy is not a confounder of the IBC gene signature.

The gene expression profiles of tumor stroma and tumor epithelium were compared between IBC and non-IBC. The cutoff point for a differentially expressed gene to be included in this analysis was $p \leq 0.01$. As shown in Figure 1, we found a significant enrichment of tumor stromal genes that distinguished IBC from non-IBC, but did not find an analogous enrichment in the tumor epithelium. The analysis suggests that distinctive gene expression patterns in the tumor stroma may contribute to the IBC phenotype.

Two shorter gene lists comprised of those genes with the most significant differences in mRNA expression between IBC and non-IBC tumors were generated. The first list shows genes that were differentially expressed only in the tumor stroma at the $p < 0.001$ significance level, as indicated by a corresponding probe set (Table II). The second list contains genes that were expressed differentially in both tumor stroma and tumor epithelium at the $p \leq 0.01$ significance level (Table III).

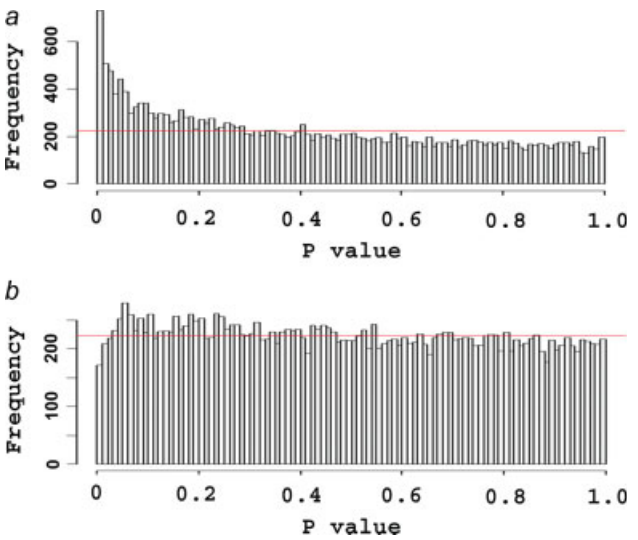

FIGURE 1 – Graphical representation of the p value distribution from the moderated *t*-test scores for the IBC *versus* non-IBC contrast in tumor stroma (*a*) and tumor epithelium (*b*) by gene frequency. The solid red line represents the expected number of p values in each of the 100 bins (223 probe sets per bin) if there are no genes systematically differentially expressed between IBC and non-IBC. Only a gene frequency that exceeds the expected number of false positives for a given p value is an indication of a true difference in the gene expression between IBC and non-IBC. The graph shows that more genes are significantly differentially expressed in tumor stroma between IBC and non-IBC tumors than expected by chance. In contrast, almost all detected differences for the IBC *versus* non-IBC comparison in the tumor epithelium appear to be false-positives. We estimate that about 30% of the differentially expressed genes in the tumor stroma contrast at the p value < 0.01 significance level are false-positives and about 70%, are truly differentially expressed.

Four genes were expressed at significantly higher levels in IBC than in non-IBC. The dual specificity phosphatase 2 (DUSP2), also called PAC-1, and laminin γ2 (LAMC2) were upregulated only in the IBC stroma (Table II) whereas endothelin-1 (EDN-1) and zinc fingers in cerebellum 1 (ZIC1) were upregulated in both IBC stroma and tumor epithelium (Table III). DUSP2 is a key positive regulator of inflammatory cell signaling in human leukocytes.[23] Laminin γ2 is a β-catenin-regulated gene that has a function in tumor invasion.[24–26] Endothelin-1 is an angiogenic factor, whose expression in breast cancer is associated with increased angiogenesis and metastasis.[27,28] ZIC1 belongs to a family of transcription factors that have been implicated in birth defects.[29]

TABLE II – STROMAL GENES DIFFERENTIALLY EXPRESSED BETWEEN IBC AND NON-IBC[1]

Gene symbol	Affymetrix ID	GenBank ID	Fold-change	*p* value[2]
Expressed at higher levels in IBC				
DUSP2	204794_at	NM_004418	1.51	9.26E−04
LAMC2	202267_at	NM_005562	1.58	3.10E−04
Expressed at lower levels in IBC				
TSPYL1	221493_at	NM_003309	0.63	9.17E−06
SLC2A10	221024_s	NM_030777	0.45	1.35E−05
UBE2J1	217826_s	NM_016021	0.62	1.53E−05
	217825_s	AF151039	0.77	1.31E−03
TTC3	210645_s	D83077	0.66	2.17E−05
	208073_x	NM_003316	0.70	1.41E−04
ITGBL1	205422_s	NM_004791	0.37	3.91E−05
UBQLN2	215884_s	NM_013444	0.66	1.05E−04
SEL1L	202061_s	NM_005065	0.66	1.10E−04
GALNT1	201722_s	NM_020474	0.66	1.16E−04
DHX29	212648_at	NM_019030	0.66	1.46E−04
LGALS8	208933_s	AI659005	0.63	1.97E−04
LUM	201744_s	NM_002345	0.57	2.20E−04
RAB2	208731_at	AU158062	0.67	2.89E−04
C6orf211	218195_at	NM_024573	0.55	4.66E−04
AGL	203566_s	NM_000645	0.67	5.32E−04
SPOCK	202363_at	NM_004598	0.63	5.38E−04
GALC	204417_at	NM_000153	0.64	6.09E−04
CAMK2N1	218309_at	NM_018584	0.60	6.41E−04
PGRMC2	213227_at	BE879873	0.66	6.51E−04
CREBL2	201990_s	NM_001310	0.63	7.69E−04
STS	203767_s	NM_000351	0.66	9.03E−04

[1]The cutoff point for genes to be included in the table is an expression change ≥1.5-fold and $p < 0.001$ for a corresponding probe set (Affymetrix ID) using moderated *t*-scores. These genes are not significantly differentially expressed in the tumor epithelium at the $p \leq 0.01$ cutoff point for our gene lists.–[2]Two-sided Welch *t*-test.

The list of genes downregulated in IBC stroma includes several previously described suppressors of metastasis, *e.g.*, SEL1L[30] and LUM or lumican,[31,32] but also osteonectin (SPOCK), which has been implicated in both promotion and inhibition of breast cancer metastasis.[33] Genes that were downregulated in both stroma and tumor include the candidate tumor suppressors ARMCX3 (also called ALEX1),[34] DUSP4[35] and PSD3 (also called EFA6R),[36] the B-cell marker and tumor suppressor BLNK,[37] and nucleobindin (NUCB2). The latter is an apoptosis and autoimmunity-associated protein that interacts with cyclooxygenase-2.[38] The lists of downregulated genes also include the steroid sulfatase (STS) and the androgen receptor (AR). Only 3 genes were expressed differentially between IBC and non-IBC in the tumor epithelium at the $p < 0.001$ significance level, and they were downregulated in IBC (data not shown). The genes were inositol 1,4,5-triphosphatase, type 1 (ITPR1), mitochondrial tumor suppressor 1 (MTUS1) and rhodanese, which is also called thiosulfate sulfurtransferase (TST).

Because Laminin γ2 was reported to be expressed in myoepithelial cells in breast tumors,[39] we investigated laminin γ2 expression in the IBC tumors by immunohistochemistry. We observed very scattered protein expression by myoepithelial cells and cancer cells in these tumors. From these data, we conclude that the detected expression of LAMC2 in the IBC tumor stroma is due to the expression of this gene in myoepithelial cells that are adjacent to the stroma and infiltrate it.

Exploratory methods such as hierarchical clustering and principal components analysis based on the expression profiles of either tumor stroma or tumor epithelium did not separate IBC cases from non-IBC cases (data not shown). These findings are consistent with other studies that observed heterogeneous expression profiles among the IBC tumors.[6,8]

Pathway analysis of gene expression patterns in IBC

Our analysis of the microarray data provided lists of genes that were differentially expressed in stroma and tumor of IBC patients when compared with non-IBC patients. Although gene lists can be very informative, single-gene effects are unlikely to cause the IBC phenotype. Rather, the cumulative effect of multiple genes on

TABLE III – GENES DIFFERENTIALLY EXPRESSED BETWEEN IBC AND NON-IBC IN TUMOR STROMA AND EPITHELIUM[1]

Gene symbol	GenBank ID	Tumor stroma		Tumor epithelium	
		Fold-change	*p* value[2]	Fold-change	*p* value[2]
Expressed at higher levels in IBC					
EDN1	NM_001955	1.76	5.03E−05	1.83	4.74E−05
ZIC1	NM_003412	1.94	3.54E−04	2.35	1.40E−04
Expressed at lower levels in IBC					
AR	NM_000044	0.67	1.79E−03	0.62	3.28E−03
ARMCX3	NM_016607	0.67	9.44E−05	0.61	1.37E−04
BLNK	NM_013314	0.57	3.39E−04	0.56	2.46E−04
Top of Form IGKV clone 25	AW404894	0.38	9.74E−03	0.34	5.07E−03
CADPS2	NM_017954	0.59	3.70E−05	0.62	6.93E−04
DUSP4	NM_001394	0.47	1.01E−02	0.38	4.61E−03
DKFZP686A01247	NM_014988	0.56	2.86E−04	0.46	2.06E−04
		0.58	2.57E−03	0.50	1.47E−03
		0.67	7.27E−04	0.64	6.32E−03
TMEM135	NM_022918	0.66	4.27E−03	0.60	2.44E−03
IGJ	NM_144646	0.46	3.26E−03	0.44	5.30E−03
MAGED2	NM_014599	0.62	1.98E−04	0.60	4.07E−03
		0.70	4.39E−04	0.71	5.35E−03
NUCB2	NM_005013	0.54	2.42E−04	0.49	4.68E−04
PECI	NM_006117	0.64	8.92E−04	0.54	1.04E−03
PSD3	NM_015310	0.43	5.61E−05	0.38	9.02E−05

[1]The cutoff point for genes to be included in the table is $p \leq 0.01$ for the moderated *t*-scores in the tumor stroma and tumor epithelium gene lists.–[2]Two-sided Welch *t*-test.

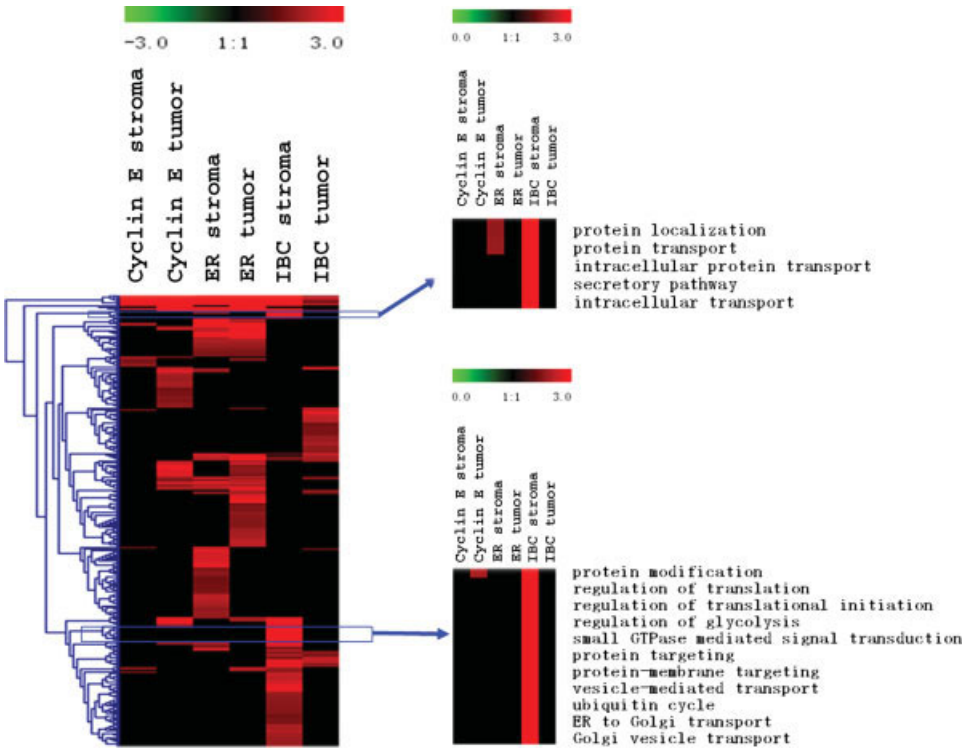

FIGURE 2 – Cluster analysis of GOBP terms for the IBC *versus* non-IBC contrast in tumor stroma (IBC stroma) and tumor epithelium (IBC tumor). For comparison, we also show the cluster analysis for the cyclin E-positive *versus* cyclin E-negative contrast and ER-positive *versus* ER-negative contrast in the non-IBC tumors. The results of our analysis are displayed as a heatmap with the red color indicating an enrichment of differentially expressed genes in a GOBP term for a particular comparison, *e.g.*, IBC *versus* non-IBC in tumor stroma. Our analysis revealed that gene expression differences cluster by GOBP term creating unique patterns of frequently affected GOBP terms for the 6 contrasts. The 2 smaller heatmaps show enlargements of 2 GOBP term clusters that contain multiple differentially expressed genes ($p \leq 0.01$; Welch *t*-test) in tumor stroma for the IBC *versus* non-IBC comparison. The cluster analysis used all GOBP terms and incorporated both the number of genes that are assigned to a GOBP term, termed list hits, and log-transformed *p* values from the Fisher's exact test to assess the significance of enrichment in GOBP terms. ER status and cyclin E expression in the tumor samples were scored as described previously.[15]

cancer-related pathways may explain the difference between disease types. We performed a comprehensive pathway analysis using the in-house WPS software[20], and the Ingenuity and MetaCore™ pathway analysis tools to identify biological networks in IBC. Pathways were annotated according to GOBP term and BioCarta pathway classifications. These 2 functional annotation methods are complementary, because GOBP uses substantially more genes for functional annotation than does BioCarta, but GOBP terms are not as detailed and well-defined as those in BioCarta pathways, and do not interpret gene–gene relationships in the context of pathways. Our database had 16,762 human genes annotated for GOBP and 1,430 genes for BioCarta pathways.

We performed cluster analysis to identify pathways with significant enrichment of genes expressed differentially between the IBC and non-IBC tumors. Analysis of the GOBP terms revealed clusters of enriched GOBP terms that created a unique pattern of frequently affected biological processes in IBC (Fig. 2). The 2 most distinctive clusters of GOBP terms for the IBC stroma contained biological processes associated with intracellular protein transport and localization, protein secretion, mRNA translation, regulation of glycolysis and GTPase signaling (Fig. 2). A more extensive list of GOBP terms for the contrast is shown in Table IV. The top-ranked terms appear to reflect biological processes that take place in the endoplasmic reticulum, and suggest differences in the endoplasmic reticulum stress response between IBC and non-IBC. Genes in these pathways were generally downregulated in IBC stroma when compared with non-IBC stroma. Supplementary Figure 1 shows those genes that are differentially expressed between IBC and non-IBC in the 5 highest-ranked GOBP terms. Many of the genes have a function in more than one of those biological processes.

The GOBP results for the tumor contrast are shown in Table V. Only 1 GOBP term, the double-strand break repair biological process, was enriched with targets in the tumor epithelium at the $p < 0.001$ significance level. Notable is the number of biological processes related to metabolism (*e.g.*, sterol, acety-CoA, aldehyde and alcohol metabolism) that had multiple genes differentially expressed between IBC and non-IBC tumor epithelium. Others have also observed an enrichment of genes in metabolism-related pathways when comparing IBC tumors and non-IBC tumors.[8]

The cluster analysis of BioCarta pathways revealed 8 pathways significantly enriched with genes differentially expressed between IBC and non-IBC contrast, and it also showed distinct patterns for tumor stroma and epithelium (Fig. 3*a*). Three pathways were enriched with genes differentially expressed in the tumor stroma, and 5 pathways were enriched with genes in the tumor epithelium. The "Rab GTPases in endocytosis" and "eukaryotic protein translation" pathways in tumor stroma and the "BRCA-1-dependent Ub-ligase activity" and "regulation of MAP kinases by dual specificity phosphatases" pathways in tumor epithelium were the top-ranked pathways for the IBC *versus* non-IBC contrast (Fig. 3*b*).

Classification of IBC phenotype using published gene signatures

We further assessed the relative contribution of the tumor stroma to the IBC phenotype using a published classifier for IBC.[6] We did not attempt to generate a classifier from our own sample set because of the limited number of IBC cases in the study. We

TABLE IV – TWENTY HIGHEST-RANKED GOBP TERMS IN THE IBC VERSUS NON-IBC CONTRAST FOR TUMOR STROMA

GOBP term	IBC list term hits[1]	IBC list total[2]	Population hits[3]	Population total[4]	Fisher's exact test *p* value
Protein localization	30	255	540	16,762	9.24E−10
Protein transport	29	255	527	16,762	2.24E−09
Intracellular protein transport	21	255	343	16,762	6.92E−08
Intracellular transport	27	255	562	16,762	1.37E−07
Secretory pathway	12	255	134	16,762	9.68E−07
ER to golgi transport	6	255	28	16,762	3.32E−06
Cell growth and/or maintenance	100	255	4559	16,762	1.80E−05
Golgi vesicle transport	7	255	65	16,762	5.70E−05
Vesicle-mediated transport	15	255	307	16,762	7.58E−05
Protein modification	50	255	1905	16,762	7.79E−05
G1/S transition of mitotic cell cycle	6	255	54	16,762	1.64E−04
Regulation of cyclin-dependent protein kinase activity	5	255	37	16,762	2.29E−04
Regulation of glycolysis	2	255	2	16,762	2.31E−04
Mitotic cell cycle	11	255	198	16,762	2.31E−04
JNK cascade	5	255	40	16,762	3.34E−04
Small GTPase-mediated signal transduction	13	255	290	16,762	5.19E−04
G2/M transition of mitotic cell cycle	5	255	44	16,762	5.24E−04
Protein kinase cascade	12	255	255	16,762	5.52E−04
Regulation of protein kinase activity	7	255	96	16,762	6.55E−04
Ubiquitin cycle	22	255	678	16,762	7.07E−04

[1]Annotated genes in a GOBP term that are differentially expressed ($p \leq 0.01$) comparing IBC with non-IBC tumors.–[2]All GOBP-annotated genes that are differently expressed in this comparison.–[3]All annotated genes in a GOBP term.–[4]All GOBP-annotated genes.

TABLE V – TEN HIGHEST-RANKED GOBP TERMS IN THE IBC VERSUS NON-IBC CONTRAST FOR TUMOR EPITHELIUM

GOBP term	IBC list term hits[1]	IBC list total[2]	Population hits[3]	Population total[4]	Fisher's exact test *p* value
Double-strand break repair	3	153	17	16,762	4.62E−04
Sterol biosynthesis	3	153	26	16,762	1.66E−03
Acetyl-CoA metabolism	2	153	8	16,762	2.24E−03
Cellular physiological process	64	153	5,150	16,762	2.28E−03
Aldehyde metabolism	2	153	10	16,762	3.55E−03
Nuclear division	6	153	165	16,762	4.08E−03
Energy pathways	8	153	280	16,762	4.22E−03
Cell proliferation	20	153	1,156	16,762	4.28E−03
Alcohol metabolism	8	153	284	16,762	4.60E−03
Response to stress	17	153	929	16,762	4.86E−03

[1]Annotated genes in a GOBP term that are differentially expressed ($p \leq 0.01$) comparing IBC with non-IBC tumors.–[2]All GOBP-annotated genes that are differently expressed in this comparison.–[3]All annotated genes in a GOBP term.–[4]All GOBP-annotated genes.

were able to map 90 of the 109 genes in the published classifier to the Affymetrix chip. PAM was applied to classify our dataset with the predefined IBC signature for the intended categories.[19] Using a threshold setting that achieved the best compensation for both training and CV error rates, we were able to classify 10 (71%) of 14 IBC cases and all the non-IBC cases correctly with the stroma signature. We achieved a correct classification of only 54% of the IBC cases (7 of 13) and 97% of the non-IBC cases (33 of 34) using the tumor epithelial signature. The data suggest that the gene expression profile of the tumor stroma is a better classifier for IBC than the gene expression profile of the tumor epithelium, and that many of the genes in the published classifier may have originated from the stromal compartment, rather than the tumor epithelium of the bulk tumors used to identify the signature.

Discussion

IBC is the most aggressive form of breast cancer and is thought to represent a distinct disease entity.[1] The genetic factors that determine whether a breast carcinoma will develop the IBC phenotype are still poorly understood. We investigated the relative contributions of the gene expression profiles in tumor stroma and epithelium to the IBC phenotype and found that IBC is more easily distinguished from non-IBC using the stromal signature than the signature of the tumor epithelium. Additional pathways analyses revealed a very distinctive clustering of differentially expressed genes into IBC-related biological processes and pathways, although the analysis of individual genes without pathway linkage demonstrated only subtle gene expression differences between IBC and non-IBC.

Others have examined gene expression in IBC. One earlier study, using differential display, showed significant differences in the expression of 2 genes, *ARHC*/*RhoC* and *WISP3*/*LIBC*, in the IBC to non-IBC comparison.[5] That observation was not confirmed in other studies.[8,40] Subsequent reports identified new sets of genes that were differentially expressed in IBC and non-IBC, including a nuclear factor-κB signature.[7,41,42] A more recent study analyzed the differences between IBC and non-IBC tumors at a pathway level and observed, as we did for the microdissected tumor epithelium, that many discriminatory pathways relate to energy and lipid metabolism, and cell proliferation.[8] That study, like ours, did not find that the expression of genes related to tissue inflammation differentiates IBC tumors from non-IBC tumors at a pathway level.

Stroma	List Hits	List Total	Population Hits	Population Total	Fisher's Exact Test *P* value
Rab GTPases in the endocytotic machinery	4	50	13	1430	7.50E-04
Eukaryotic protein translation	3	50	9	1430	2.91E-03
Tumor epithelium					
BRCA1-dependent Ub-ligase activity	2	28	16	1430	3.75E-02
Regulation of MAP kinase pathway by DUSP	2	28	10	1430	3.84E-02

FIGURE 3 – Cluster analysis of 8 BioCarta pathways for the IBC *versus* non-IBC contrast in tumor stroma (IBC stroma) and tumor epithelium (IBC tumor). The results of the analysis are displayed in a heatmap (*a*). The red color indicates an enrichment of differentially expressed genes in a pathway for a particular comparison, *e.g.*, IBC *versus* non-IBC in tumor stroma. The pathways were selected because they have both an enrichment of genes that are differentially expressed in the IBC *versus* non-IBC comparison and a distinct pattern for tumor stroma and tumor. For example, multiple differentially expressed genes cluster in 3 pathways in tumor stroma, *e.g.*, the *SREBP*, eukaryotic protein translation, and *RAP* GTPases pathways, but do not cluster in these pathways in the tumor epithelium. We used 2 related parameters, the log-transformed *p* values and the list hits methods, to display an enrichment of genes by pathways. ERAD is the abbreviation of endoplasmic reticulum-associated degradation. (*b*) List of BioCarta pathways that have the most significant enrichment of differentially expressed genes between IBC and non-IBC. A one-sided Fisher's exact test for over-represented pathways was used for significance testing.

Differences in methodology may explain some of the differences among the gene lists generated in the various studies of IBC. Stage-matching of IBC and non-IBC patients, as done in some studies, can introduce a bias to the detected differences in the gene expression profiles, as previously discussed.[7,43] However, we stress here another important limitation of previous gene expression studies of IBC: the use of whole tissue samples. The tumor-to-stroma ratio is generally lower in IBC than it is in non-IBC.[43] Furthermore, IBC is characterized by disseminated growth of the primary tumor and secondary changes to the breast tissue that are attributable to blockage of lymphatics by tumor infiltrates.[2] Those characteristics can lead to wide variations in the composition of whole tissue resections and introduce heterogeneity into the gene expression profiles, increasing the likelihood of either false-positive findings or gene signatures that are specific to a sample set.

We, therefore, studied the gene expression differences between IBC and non-IBC in microdissected samples. Microdissection allows the collection of relatively pure, or at least considerably enriched, populations of tumor stromal and epithelial cells. As a consequence, we could detect quite subtle gene expression differences that might have been obscured if we had analyzed bulk tumor. Nevertheless, our analysis did not find statistically robust differences in the global gene expression between IBC and non-IBC tumor epithelia. That finding probably reflects high overall variability in gene expression and genetic heterogeneity of IBC. Overall, our results are consistent with the evidence that IBC and non-IBC tumors originate from the same cell subtypes.[44,45] Significant differences in gene expression were observed for some tumor epithelial genes in our study, but the findings have to be interpreted with caution because of the statistical multiple comparisons problem.

In contrast to the evaluation of the IBC gene signature in the tumor epithelium, our assessment of the microdissected tumor stroma revealed a number of very significant differences between IBC and non-IBC at both the gene and pathway level. Most of the genes were modestly differentially expressed. Many of the gene differences clustered in particular biological processes and pathways, and the enrichment in these processes and pathways was highly statistically significant. A further extension of our pathway analysis to other important markers of disease outcome, *e.g.* tumor ER status and cyclin E overexpression, indicated that the observed expression differences in these particular biological processes and pathways could be unique to the IBC to non-IBC comparison. Most of those distinguishing pathways were associated with intracellular trafficking, localization, modification and secretion of proteins with a particular involvement of *RAB* GTPases among other genes. The biological theme of these pathways alteration points to a modified endoplasmic reticulum stress response in IBC stroma. The decreased expression of a large number of genes in the IBC stroma that facilitate the stress response may indicate a mechanism of protection. Indeed, it has been found that endoplasmic reticulum stress is anti-oncogenic and induces premature senescence in primary human cells,[46] and an attenuation of this pathway in IBC stroma may preserve the integrity of stromal cell function in stress situations. The stromal signature may also reflect an adaptation of the stroma to the stress associated with the hyperproliferation of IBC.

Gene signatures derived from primary tumors have been shown to predict response to therapy, distant metastasis and poor survival.[47–50] Patterns of gene expression in breast tumors have also been associated with molecularly distinct subtypes of locally advanced breast cancer, tumor ER status and tumor grade.[51–55] We evaluated the relative contribution of the stromal signature to the IBC phenotype using an established signature from another study.[6] This approach also served as cross-validation of our classification criteria for IBC. We achieved a good classification of IBC with the gene expression profile from tumor stroma, but not with that from the tumor epithelium. Our ability to classify IBC with the stromal signature appeared to be as good as that achieved in the original report using bulk tumor specimens.[6] This finding that the classifier performed well with the gene expression profile of microdissected stroma suggests that gene expression in the tumor stroma contributes to IBC.

This is the first study to make use of systematically microdissected breast tumor epithelium and tumor stroma for gene expression profiling. Despite the advantages of more rigorous histological sampling enabled by LCM, the approach has limitations. LCM is labor intensive and not suitable for the analyses of large sample sets. We studied 15 IBC and 35 non-IBC cases. This sample size is large for LCM, but is smaller than some other studies of IBC that used bulk tissue. Because of this limitation, we may have failed to detect some differences between IBC and non-IBC tumors that would have been significant in a larger samples set. A second limitation relates to the low levels of total RNA that can feasibly be collected using LCM. An additional step of linear amplification was required to obtain sufficient amounts of RNA for hybridization. We and others[17,55] evaluated the effect of linear amplification on the gene expression profile and found that it does not introduce a bias to the computed relative gene expression levels. A final limitation of our analysis stems from the fact that IBC patients were given neoadjuvant chemotherapy more often than were non-IBC patients. Given the poorly defined anatomical location of most tumors in IBC patients, the vast majority of patients with that type of breast cancer received neoadjuvant treatment before surgery, whereas most patients with non-IBC received chemotherapy only after the surgical resection of the specimens that we studied. When we asked whether presurgical therapy had introduced a bias to the gene expression profile, we found only a modest, nonconfounding effect of neoadjuvant therapy on the expression differences between IBC and non-IBC tumors. This finding is consistent with previous observations by others that

therapy has only modest effects on the intrinsic gene expression profile of breast tumors.[22,51,56]

In conclusion, the present study made the novel observation that gene expression profiles of IBC stroma are different from those of non-IBC stroma. The study also identified molecular pathways that could be functionally different and significant in stromal cells of IBC. Those findings, as well as the comprehensive databases of gene expression in microdissected IBC and non-IBC tumors, may provide opportunities to better understand the clinical behavior of IBC, including its poor prognosis, and may help in intervention strategy.

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References

1. Walshe JM, Swain SM. Clinical aspects of inflammatory breast cancer. Breast Dis 2005;22:35–44.
2. Kleer CG, van Golen KL, Merajver SD. Molecular biology of breast cancer metastasis. Inflammatory breast cancer: clinical syndrome and molecular determinants. Breast Cancer Res 2000;2:423–9.
3. McCarthy NJ, Yang X, Linnoila IR, Merino MJ, Hewitt SM, Parr AL, Paik S, Steinberg SM, Hartmann DP, Mourali N, Levine PH, Swain SM. Microvessel density, expression of estrogen receptor α, MIB-1, p53, and c-erbB-2 in inflammatory breast cancer. Clin Cancer Res 2002;8:3857–62.
4. Levine PH, Steinhorn SC, Ries LG, Aron JL. Inflammatory breast cancer: the experience of the surveillance, epidemiology, and end results (SEER) program. J Natl Cancer Inst 1985;74:291–7.
5. van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res 1999;5:2511–19.
6. Bertucci F, Finetti P, Rougemont J, Charafe-Jauffret E, Nasser V, Loriod B, Camerlo J, Tagett R, Tarpin C, Houvenaeghel G, Nguyen C, Maraninchi D, et al. Gene expression profiling for molecular characterization of inflammatory breast cancer and prediction of response to chemotherapy. Cancer Res 2004;64:8558–65.
7. Van Laere S, Van der Auwera I, Van den Eynden GG, Fox SB, Bianchi F, Harris AL, van Dam P, Van Marck EA, Vermeulen PB, Dirix LY. Distinct molecular signature of inflammatory breast cancer by cDNA microarray analysis. Breast Cancer Res Treat 2005;93:237–46.
8. Nguyen DM, Sam K, Tsimelzon A, Li X, Wong H, Mohsin S, Clark GM, Hilsenbeck SG, Elledge RM, Allred DC, O'Connell P, Chang JC. Molecular heterogeneity of inflammatory breast cancer: a hyperproliferative phenotype. Clin Cancer Res 2006;12:5047–54.
9. Kleer CG, van Golen KL, Braun T, Merajver SD. Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol 2001;14:458–64.
10. Sugiyama Y, Farrow B, Murillo C, Li J, Watanabe H, Sugiyama K, Evers BM. Analysis of differential gene expression patterns in colon cancer and cancer stroma using microdissected tissues. Gastroenterology 2005;128:480–6.
11. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420: 860–7.
12. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335–48.
13. Schor SL, Haggie JA, Durning P, Howell A, Smith L, Sellwood RA, Crowther D. Occurrence of a fetal fibroblast phenotype in familial breast cancer. Int J Cancer 1986;37:831–6.
14. Hunter KW, Crawford NP. Germ line polymorphism in metastatic progression. Cancer Res 2006;66:1251–4.
15. Boersma BJ, Howe TM, Goodman JE, Yfantis HG, Lee DH, Chanock SJ, Ambs S. Association of breast cancer outcome with status of p53 and MDM2 SNP309. J Natl Cancer Inst 2006;98:911–19.
16. Chang S, Parker SL, Pham T, Buzdar AU, Hursting SD. Inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program of the National Cancer Institute, 1975–1992. Cancer 1998;82:2366–72.
17. Dumur CI, Garrett CT, Archer KJ, Nasim S, Wilkinson DS, Ferreira-Gonzalez A. Evaluation of a linear amplification method for small samples used on high-density oligonucleotide microarray analysis. Anal Biochem 2004;331:314–21.
18. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001;98:5116–21.
19. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. Proc Natl Acad Sci USA 2002;99:6567–72.
20. Yi M, Horton JD, Cohen JC, Hobbs HH, Stephens RM. WholePathwayScope: a comprehensive pathway-based analysis tool for high-throughput data. BMC Bioinformatics 2006;7:30.
21. Prueitt RL, Boersma BJ, Howe TM, Goodman JE, Thomas DD, Ying L, Pfiester CM, Yfantis HG, Cottrell JR, Lee DH, Remaley AT, Hofseth LJ, et al. Inflammation and IGF-I activate the Akt pathway in breast cancer. Int J Cancer 2007;120:796–805.
22. Korn EL, McShane LM, Troendle JF, Rosenwald A, Simon R. Identifying pre-post chemotherapy differences in gene expression in breast tumours: a statistical method appropriate for this aim. Br J Cancer 2002;86:1093–6.
23. Jeffrey KL, Brummer T, Rolph MS, Liu SM, Callejas NA, Grumont RJ, Gillieron C, Mackay F, Grey S, Camps M, Rommel C, Gerondakis SD, et al. Positive regulation of immune cell function and inflammatory responses by phosphatase PAC-1. Nat Immunol 2006;7:274–83.
24. Hlubek F, Jung A, Kotzor N, Kirchner T, Brabletz T. Expression of the invasion factor laminin γ2 in colorectal carcinomas is regulated by β-catenin. Cancer Res 2001;61:8089–93.
25. Yamamoto H, Itoh F, Iku S, Hosokawa M, Imai K. Expression of the γ(2) chain of laminin-5 at the invasive front is associated with recurrence and poor prognosis in human esophageal squamous cell carcinoma. Clin Cancer Res 2001;7:896–900.
26. Katayama M, Sanzen N, Funakoshi A, Sekiguchi K. Laminin γ2-chain fragment in the circulation: a prognostic indicator of epithelial tumor invasion. Cancer Res 2003;63:222–9.
27. Yin JJ, Mohammad KS, Kakonen SM, Harris S, Wu-Wong JR, Wessale JL, Padley RJ, Garrett IR, Chirgwin JM, Guise TA. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci USA 2003;100:10954–9.
28. Wulfing P, Diallo R, Kersting C, Wulfing C, Poremba C, Rody A, Greb RR, Bocker W, Kiesel L. Expression of endothelin-1, endothelin-A, and endothelin-B receptor in human breast cancer and correlation with long-term follow-up. Clin Cancer Res 2003;9:4125–31.
29. Grinberg I, Millen KJ. The ZIC gene family in development and disease. Clin Genet 2005;67:290–296.
30. Biunno I, Cattaneo M, Orlandi R, Canton C, Biagiotti L, Ferrero S, Barberis M, Pupa SM, Scarpa A, Menard S. SEL1L a multifaceted protein playing a role in tumor progression. J Cell Physiol 2006;208: 23–38.
31. Vuillermoz B, Khoruzhenko A, D'Onofrio MF, Ramont L, Venteo L, Perreau C, Antonicelli F, Maquart FX, Wegrowski Y. The small leucine-rich proteoglycan lumican inhibits melanoma progression. Exp Cell Res 2004;296:294–306.
32. Troup S, Njue C, Kliewer EV, Parisien M, Roskelley C, Chakravarti S, Roughley PJ, Murphy LC, Watson PH. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. Clin Cancer Res 2003;9:207–14.
33. Koblinski JE, Kaplan-Singer BR, VanOsdol SJ, Wu M, Engbring JA, Wang S, Goldsmith CM, Piper JT, Vostal JG, Harms JF, Welch DR, Kleinman HK. Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. Cancer Res 2005;65:7370–7.
34. Kurochkin IV, Yonemitsu N, Funahashi SI, Nomura H. ALEX1, a novel human armadillo repeat protein that is expressed differentially in normal tissues and carcinomas. Biochem Biophys Res Commun 2001;280:340–7.
35. Armes JE, Hammet F, de Silva M, Ciciulla J, Ramus SJ, Soo WK, Mahoney A, Yarovaya N, Henderson MA, Gish K, Hutchins AM, Price GR, et al. Candidate tumor-suppressor genes on chromosome arm 8p in early-onset and high-grade breast cancers. Oncogene 2004; 23:5697–702.

36. Pils D, Horak P, Gleiss A, Sax C, Fabjani G, Moebus VJ, Zielinski C, Reinthaller A, Zeillinger R, Krainer M. Five genes from chromosomal band 8p22 are significantly down-regulated in ovarian carcinoma: N33 and EFA6R have a potential impact on overall survival. Cancer 2005;104:2417–29.
37. Flemming A, Brummer T, Reth M, Jumaa H. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. Nat Immunol 2003;4:38–43.
38. Ballif BA, Mincek NV, Barratt JT, Wilson ML, Simmons DL. Interaction of cyclooxygenases with an apoptosis- and autoimmunity-associated protein. Proc Natl Acad Sci USA 1996;93:5544–9.
39. Pyke C, Salo S, Ralfkiaer E, Romer J, Dano K, Tryggvason K. Laminin-5 is a marker of invading cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. Cancer Res 1995; 55:4132–9.
40. Bieche I, Lerebours F, Tozlu S, Espie M, Marty M, Lidereau R. Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. Clin Cancer Res 2004;10: 6789–95.
41. Dressman HK, Hans C, Bild A, Olson JA, Rosen E, Marcom PK, Liotcheva VB, Jones EL, Vujaskovic Z, Marks J, Dewhirst MW, West M, et al. Gene expression profiles of multiple breast cancer phenotypes and response to neoadjuvant chemotherapy. Clin Cancer Res 2006;12:819–26.
42. Van Laere SJ, Van der Auwera I, Van den Eynden GG, Elst HJ, Weyler J, Harris AL, van Dam P, Van Marck EA, Vermeulen PB, Dirix LY. Nuclear factor-kappaB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-kappaB DNA-binding. Clin Cancer Res 2006;12:3249–56.
43. Van der Auwera I, Van Laere SJ, Van den Eynden GG, Benoy I, van Dam P, Colpaert CG, Fox SB, Turley H, Harris AL, Van Marck EA, Vermeulen PB, Dirix LY. Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin Cancer Res 2004;10:7965–71.
44. Bertucci F, Finetti P, Rougemont J, Charafe-Jauffret E, Cervera N, Tarpin C, Nguyen C, Xerri L, Houlgatte R, Jacquemier J, Viens P, Birnbaum D. Gene expression profiling identifies molecular subtypes of inflammatory breast cancer. Cancer Res 2005;65:2170–8.
45. Van Laere SJ, Van den Eynden GG, Van der Auwera I, Vandenberghe M, van Dam P, Van Marck EA, van Golen KL, Vermeulen PB, Dirix LY. Identification of cell-of-origin breast tumor subtypes in inflammatory breast cancer by gene expression profiling. Breast Cancer Res Treat 2006;95:243–55.
46. Denoyelle C, Abou-Rjaily G, Bezrookove V, Verhaegen M, Johnson TM, Fullen DR, Pointer JN, Gruber SB, Su LD, Nikiforov MA, Kaufman RJ, Bastian BC, et al. Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. Nat Cell Biol 2006;8:1053–63.
47. Ma XJ, Wang Z, Ryan PD, Isakoff SJ, Barmettler A, Fuller A, Muir B, Mohapatra G, Salunga R, Tuggle JT, Tran Y, Tran D, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell 2004;5:607–16.
48. Van de Vijver MJ, He YD, Van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.
49. van 't Veer LJ, Dai H, Van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der KK, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.
50. Chang HY, Nuyten DS, Sneddon JB, Hastie T, Tibshirani R, Sorlie T, Dai H, He YD, Van't Veer LJ, Bartelink H, Van De RM, Brown PO, et al. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA 2005;102:3738–43.
51. Perou CM, Sorlie T, Eisen MB, Van De RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
52. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, Van De RM, Jeffrey SS, Thorsen T, Quist H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001; 98:10869–74.
53. Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 2001;61:5979–84.
54. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci USA 2003;100:10393–98.
55. Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, Payette T, Pistone M, Stecker K, Zhang BM, Zhou YX, Varnholt H, et al. Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci USA 2003;100:5974–9.
56. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 2003;100: 8418–23.